

AD _____

Award Number: W81XWH-10-1-0253

TITLE: The Role of Megakaryocytes in Breast Cancer Metastasis to Bone

PRINCIPAL INVESTIGATOR: Andrea M. Mastro

CONTRACTING ORGANIZATION:

Pennsylvania State University
University Park, PA 16802

REPORT DATE: May 2014

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

x Approved for public release; distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE May 2014		2. REPORT TYPE Final		3. DATES COVERED 15 Apr 10-14 Apr 14	
4. TITLE AND SUBTITLE The Role of Megakaryocytes in Breast Cancer Metastasis to Bone				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-10-1-0253	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Andrea M. Mastro, Walter Jackson III, Paige N. Chandler E-Mail: a36@psu.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) The Pennsylvania State University University Park, Pennsylvania 16802				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Hypothesis: megakaryocytes (MKs) contribute to the growth of metastatic breast cancer in the bone either by preparing a niche and/or by responding to the cytokines of the marrow resulting from the interaction of the cancer with cells of the marrow. We found that MKs increased in the femurs of mice bearing MDA-MB-231 human cancer. We compared MKs in femurs of nude mice inoculated with cancer cells into the mammary gland (non-metastasizing) with (intracardiac injection) bone metastasizing. Immunohistochemistry and counting of vonWillibrand factor+, multinucleated cells were used to determine MK numbers. Blood platelets, serum levels of thrombopoietin (TPO) and SDF-1 were measured. In another model, mouse mammary tumor cells (4T1.2 metastatic) or (67NR, non-metastatic) were injected into the mammary glands, and femurs and spleens assayed over time. The MK increased only in the metastatic model suggesting that the effect was local to the bone. However, the increase of MK was greatest in the spleen, extramedullary hematopoiesis. Results of an in vitro complementary study indicated that both osteoblasts and breast cancer cells together produced factor(s) that increase MK differentiation. In the meantime TPO-/- mouse embryos were regenerated and mice were backcrossed to Balb/c so that metastasis could be determined in MK deficient mice. The TPO-/- mice appear to be more susceptible to metastasis than the wildtype, with metastases appearing more quickly and spreading further after inoculation.					
15. SUBJECT TERMS Megakaryocytes, breast cancer, bone metastasis					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES 24	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	22
Reportable Outcomes.....	22
Conclusion.....	23
References.....	25
Appendices.....	None

INTRODUCTION

Metastasis to bone is a common sequela of breast cancer. Why breast and certain other solid tumors prefer to metastasize to bone versus other organs is not clear. While examining sections of femurs of mice that had been inoculated with metastatic human breast cancer cells, we observed that megakaryocyte (MK) numbers were significantly increased in the bone marrow of mice with cancer compared with non-tumor bearing mice [1]. We could find no reports of a direct role for MKs in metastatic cancer but many indirect connections. For example, MKs are the source of platelets which release growth and angiogenic factors, and contribute to basement membrane proteolysis [2]. Thromboembolism is one of the most common causes of death in human cancer patients. MKs differentiate in the endosteal niche, the same niche where cancer cells home. In addition MKs produce many characteristic osteoblastic molecules such as RANKL and OPG that can regulate osteoclast activity. This information taken together led to the hypothesis that MKs contribute to growth of breast cancer cells in the bone either by preparing a niche and/or by responding to the cytokine microenvironment of the marrow that results from the interaction of the cancer cells and the osteoblasts/stromal cells. Mouse models, xenograft and syngeneic, were used to compare MKs in femurs under conditions of metastasis or non-metastasis. In the syngeneic model we found a large increase in the spleen, extramedullary hematopoiesis. Thrombopoietin (TPO^{-/-}) knockout mice were generated and used to test metastasis in mice with a MK deficiency. Contrary to expectations, TPO^{-/-} mice showed greater metastasis than wildtype mice.

BODY

Task 1. Begin the process of creating TPO^{-/-} mice on a Balb/c background.

We began this task immediately upon receipt of the grant. We had received permission from Genentech to obtain frozen embryos of TPO^{-/-} mice which were on a C57Blk6 background. Genentech no longer maintains the line of mice but had stored frozen embryos at Jackson Laboratories. We originally proposed to have the embryos shipped here and the work would be done at Penn State. However, because Jackson Laboratory already had the frozen embryos, it made technical and economical sense to engage them to regenerate the frozen embryos and to mate the mice to transfer the TPO^{-/-} from the C57Blk6 background onto the Balb/c background. It took Jackson three years to successfully regenerate the frozen embryos and backcross them to Balb/c. On April 3, 2013 we received from Jackson Laboratory 2 breeding pairs heterozygote (TPO^{-/+}) on a Balb/c background (99.25%). We also obtained 2 female and one male wild type Balb/c. We immediately began breeding these mice to obtain TPO^{-/-} female mice. We obtained a no-cost extension so that we would be able to test the mice for metastasis (see Task 5).

Tasks 2-3. Megakaryocytes and bone metastases in a xenograft model, a comparison of megakaryocytes in femurs of mice with bone metastatic cancer compared with cancer in the mammary gland.

We optimized immunohistochemical procedures for the detection of von Willebrand factor, and determined that this proceeded gave better detection of megakaryocytes than did H&E staining. We also carried out a pilot experiment to compare two MDA-MB-231 cell variants, 1833TR-luc and SCP2-luc, both from the laboratory of Dr. Joan Massague [3], to test how they grew in the mammary gland (intramammary injection) and in the bone (intracardiac injection). The 1833TR

grew both in the mammary glands and in the bone. The SCP2 grew only in the bone. Therefore the 1833TR were used for the next set of experiments in which MK were compared in metastatic and non-metastatic conditions.

2a. Quantification of megakaryocytes in the femurs of mice inoculated with MDA-MB-231 (1833TR-luc, luciferase) cells in the left ventricle of the heart.

This experiment was carried out to determine if the cancer metastases appeared in the bone prior to or following the increase in the megakaryocytes.

1 Platelet counts

Six mice per group were inoculated in the left ventricle of the heart with 1833TR-luc cells (10^5 in PBS) or with 100 μ l PBS. The mice were imaged with IVIS immediate after inoculation and again prior to sacrifice at 1, 4, 10, 20 and 30 days after inoculation of the tumor cells. Blood samples were obtained at the time of sacrifice and analyzed for complete blood counts with an automated Hemavet 950 instrument. Platelet counts varied considerably from animal to animal (Figure 1). There was a trend in the average counts towards an increase in the mice with intracardiac inoculation of the 1833 cells. However, there were no statistically significant differences among groups.

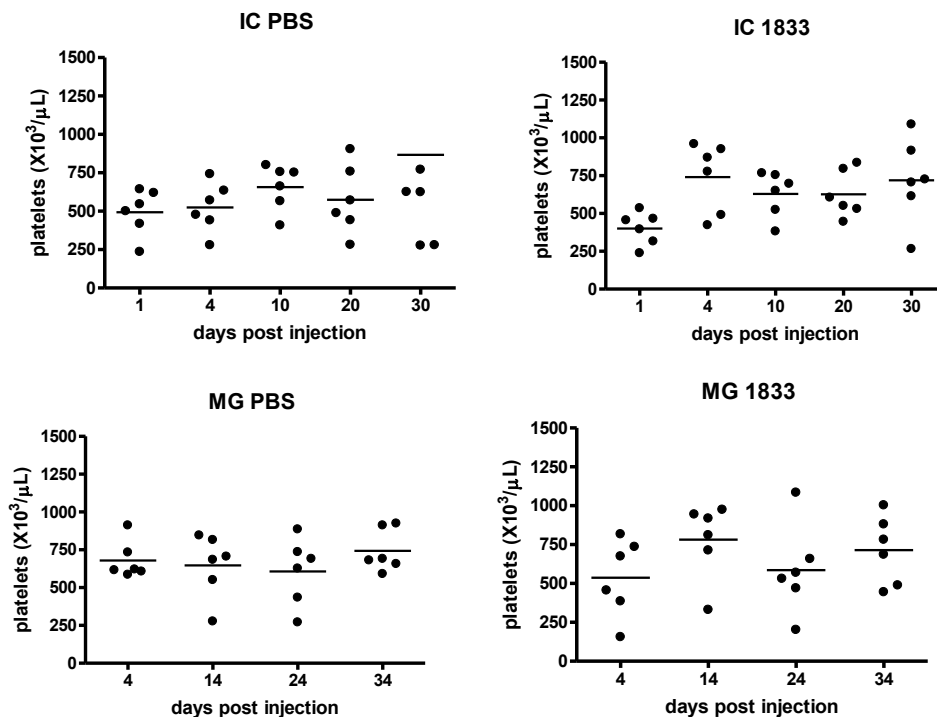


Figure 1. Blood platelet counts from mice following inoculation of MDA-MB-231 (1833TR-luc) cells or PBS into the left ventricle of the heart (intracardiac IC) or into the mammary gland (MG). Blood was collected at the time of sacrifice as indicated. Shown are the means \pm SD for six mice per group. Note that one point, PBS 30 min, was off scale and is not depicted to avoid compressing the other values, but the value was included in the calculation.

TPO and SDF-1

Thrombopoietin (TPO) is a glycoprotein hormone that stimulates the production and differentiation of MKs. Stromal cell-derived factor-1 (SDF-1) is a chemokine produced by endothelial cells in the bone and serves to attract MKs to the blood vessels in the bone. Sera from the mice were analyzed for the presence of TPO and SDF-1 (Figure 2). There were no significant differences among the groups.

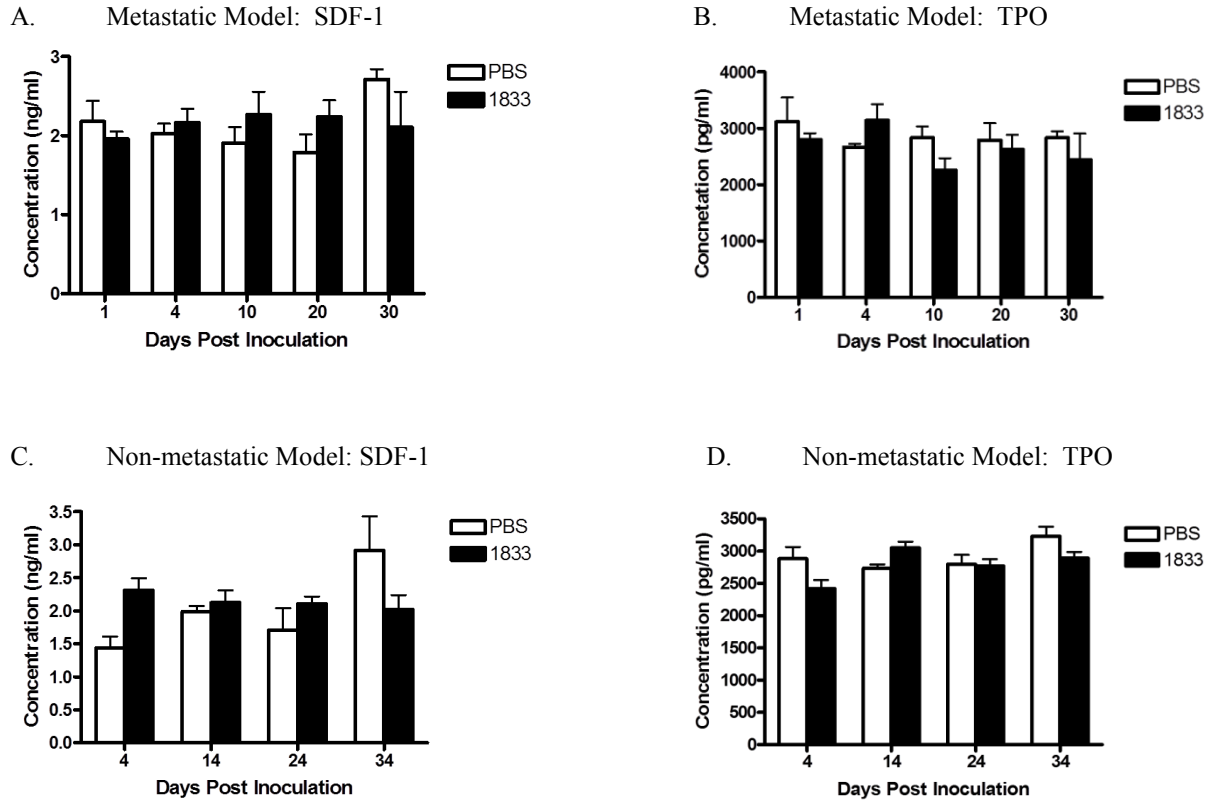


Figure 2. Serum levels of SDF-1 and TPO in mice inoculated into the left ventricle of the heart (A, B, metastatic model) or mammary gland (C, D, non-metastatic model) with MDA-MB-231, 1833, human breast cancer cells or with PBS. Serum samples, taken at the times indicated, were analyzed by ELISA. There were 6 mice per group. The average reported range for SDF-1 levels in mice is 1.46-6.84ng/mL; the average values for TPO is in the range of 2972-6645pg/mL.

3 Megakaryocyte numbers

Femurs were fixed in 4% paraformaldehyde, decalcified in EDTA and embedded in paraffin. Longitudinal sections (10 microns) were prepared from areas throughout each bone. For immunohistochemistry, the sections on Shandon Superfrost® Plus slides, were dewaxed and antigen retrieval was carried out with a 15 min treatment at 37° with 0.5% trypsin in a humidified chamber. To reduce endogenous peroxidase activity, the sections were incubated with 3% H₂O₂ for 45 min. The sections were incubated with 10% donkey serum in PBS for 2 hr, incubated overnight with a primary rabbit antibody (10 µg/ml, ABCAM) to von Willebrand factor, washed and incubated with a secondary antibody, biotinylated donkey anti-rabbit (1:1000

in 10% donkey serum) for 2 hr. Antigens were visualized with avidin-conjugated horseradish peroxidase and DAB substrate (Vector Labs). Sections were counterstained with hematoxylin. MKs were quantified manually by counting the von Willibrand positive, large, multinucleated cells in 30 fields in each of two femurs per mouse. Counting was carried out by at least two individuals, in a blinded fashion.

The number of MKs per field increased with time in the mice given intracardiac inoculations. By 30 days there were on average about twice as many MKs per field in the mice inoculated with tumors than in the mice inoculated with PBS (Figure 3A). However, at the earlier times, the MK numbers in the femurs of the tumor bearing mice were the same as in those inoculated with PBS. Therefore, these data supported the model in which MKs increase due to the presence of the tumor cells and not vice a versa.

The mice inoculated in the mammary gland showed primary tumor growth but no metastasis. The megakaryocytes in the femurs did not increase with time (Figure 3B). These data support the idea that the effect of the cancer cells on the differentiation of the megakaryocytes was not systemic.

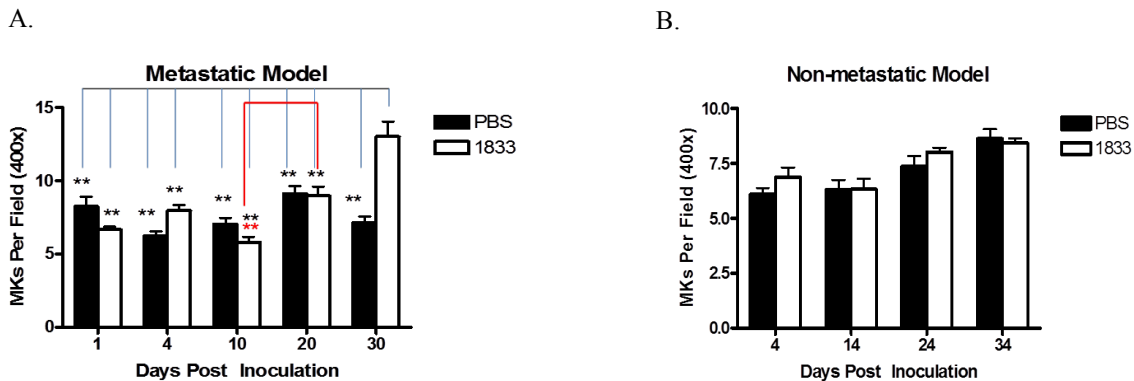


Figure 3. Megakaryocyte numbers in the femurs of mice inoculated into the left ventricle of the heart (A, metastatic model) or into the mammary gland (B, non-metastatic model) with MDA-MB-231, 1833, human breast cancer cells or with PBS. A. Femurs from 6 mice per cohort, harvested at the days indicated following intracardiac inoculation, were fixed and prepared for IHC. MKs (vWf+) were counted in 30 fields per femur, 2 femurs per mouse. Shown are the average number of MKs per 400x field +/- S.E.M. $*=p<0.05$; $**=p<0.001$, compared with 1833 at 30 days. $**=p<0.001$, compared with 1833 at 20 Days. **B.** Femurs from 6 mice per cohort, harvested at the days indicated following mammary gland injection, were fixed and prepared for IHC. MKs (vWf+) were counted in 30 fields per femur, 2 femurs per mouse. Shown is the average number of MKs per 400x field +/- S.E.M. None of the values were statistically significantly different from the others.

4 Tumor burden

The mice were imaged for the presence of the luciferase expressing tumor cells at the time of sacrifice, days 4, 14, 24 and 34 (Figure 4). Tumor burden was estimated from the IVIS images by the Living Image® version 2.60.1/Igor Pro 4.09A (Figure 4A). MKs per femur was proportional to the tumor burden (Figure 4B) suggesting that megakaryopoiesis correlated with the presence of the tumor.

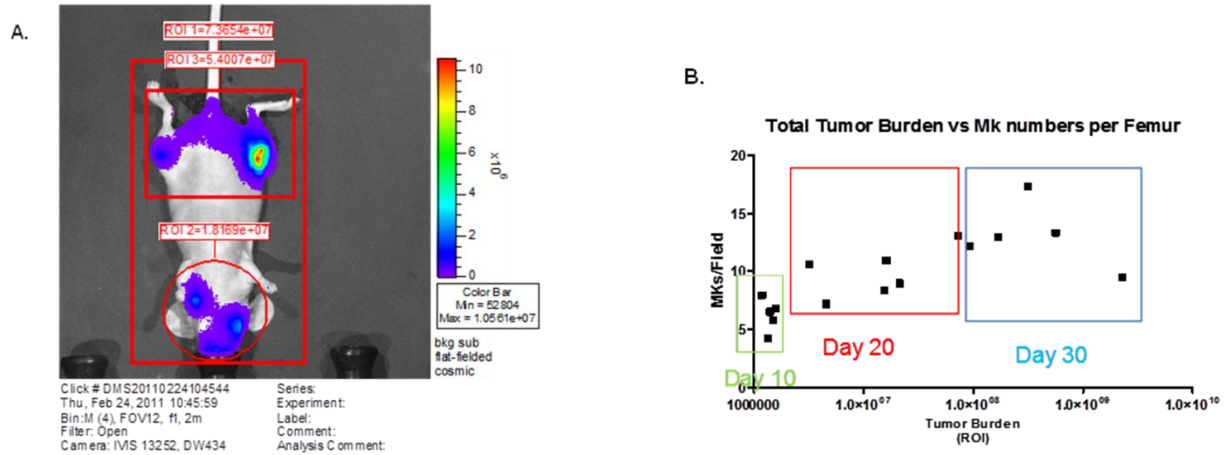
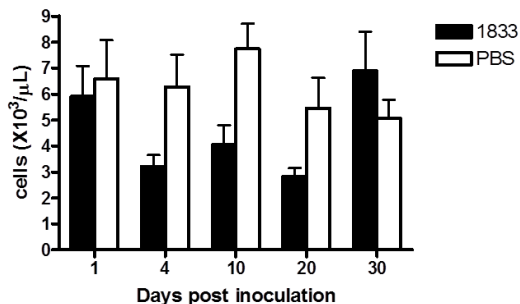


Figure 4. Tumor burden in mice following intracardiac inoculation. A. The total tumor burden as defined by the amount of bioluminescence (photon flux) measured from the IVIS images and delineated in the region of Interest determined by using Living Image(R), version 2.60.1/Igor Pro 4.09A. B. Tumor burden was plotted vs MK numbers in the femurs of mice with metastases. $n=16$ total mice with metastasis (days 10, 20, 30). Pearson correlation of = 0.680, P -Value < 0.005.

5 White Blood Cell Count

Although we had found no difference in platelet counts, we asked if there were any changes in the white blood counts (Figure 5). The white blood cell counts were all within the normal range and were not significantly different among the treatment or time groups.

A. Intracardiac Model



B. Mammary Gland Model

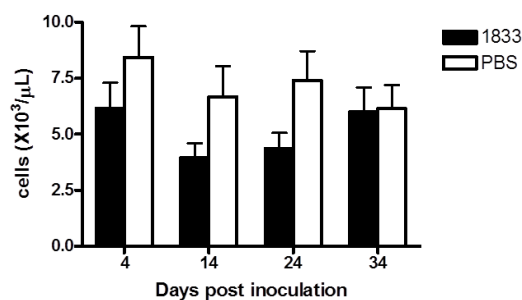


Figure 5. White Blood Cell counts from mice following inoculation of MDA-MB-231 (1833TR-luc) cells or PBS into the left ventricle of the heart (intracardiac) or into the mammary gland (MG). Blood was collected at the time of sacrifice and processed with a Hemavet 950 instrument. Shown are the means \pm SD for six mice per group.

Task 4. Syngeneic mouse model

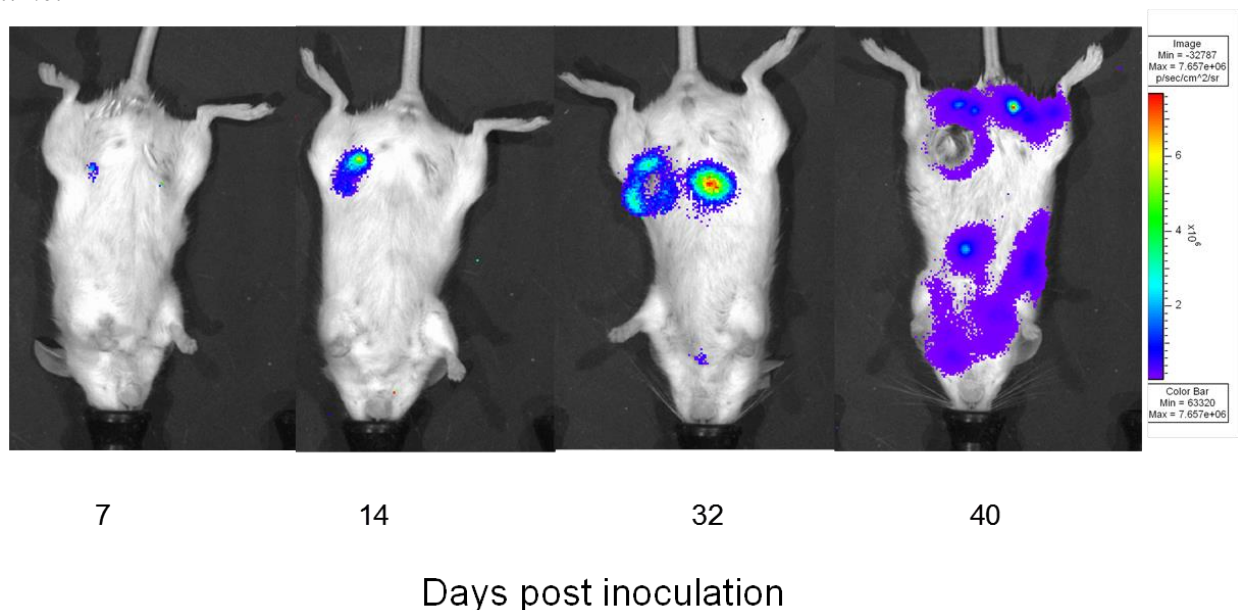
The purpose of this task was to compare the changes in MK numbers in the femurs of mice injected in the mammary glands with metastatic and non-metastatic cancer cells of the 4T1 murine series. This is a syngeneic model; therefore, the cancer and host cytokines and factors are of the same species. Furthermore, the metastasis progresses from the orthotopic site.

We obtained luciferase expressing 4T1.2 and 67NR cells which allows us to IVIS image live animals. In a preliminary study we counted MKs in the bones of Balb/c mice that had been injected with 4T1.2 cells as part of another experiment. Thirty days after the mammary gland injection with 4T1.2, the mice showed numerous and large metastases. The femurs from these animals had about twice the number per field compared with femurs from mice injected with PBS. These preliminary results indicate that the increase in MKs was not limited to the xenograft model.

1. Metastasis

We have now completed a more extensive study with the 4T1.2 model. Six mice per group were inoculated with 4T1.2 cells (5×10^5 /mammary gland). The primary tumors grew and metastasized to the skeleton and to other organs (Figure 6).

Figure 6. IVIS images of BALB/C mice taken at 7, 14, 32, and 40 days after mammary gland inoculation with 4T1.2-Luc mammary tumor cells. Six mice per group were inoculated in the mammary gland with 4T1.2 or with PBS, as a control. The PBS inoculated mice were not imaged. Shown are the images from one mouse inoculated with 4T1.2-luc and followed over time.



2. Megakaryocyte numbers

At 7, 14, 32 and 40 days post inoculation the mice, (6 per group) were sacrificed. The femurs were harvested, fixed and prepared for paraffin embedding, sectioning and immunohistochemical staining for von Willebrand factor (Figure 7). Contrary to our expectations, we saw no significant difference in megakaryocyte numbers in the femurs of mice inoculated with 4T1.2 and those with PBS.

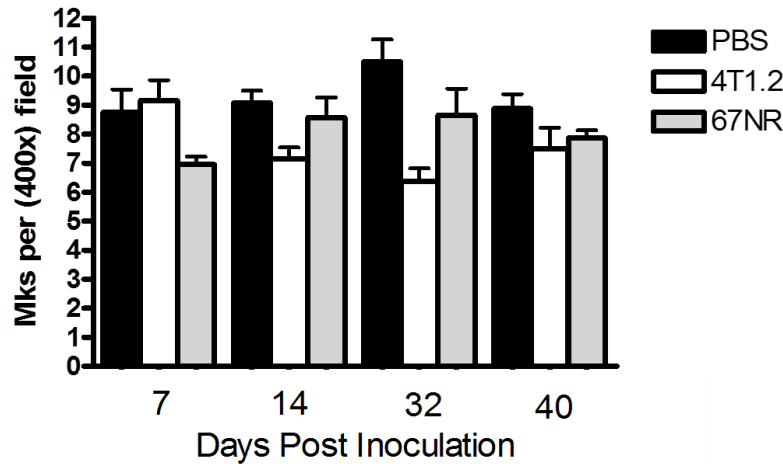


Figure 7. Megakaryocytes in femurs of mice inoculated with 4T1.2 cell, 67NR cells or PBS.

Femurs from 6 mice per cohort were harvested 7, 14, 32, or 40 days following mammary gland inoculation of 4T1.2, 67NR or PBS, fixed and prepared for IHC. MKs (vWf+) were counted in 30 fields per femur, 2 femurs per mouse. Shown are the average numbers of MKs per 400x field \pm S.E.M. The values were not statistically different among groups or over time.

However, we noted that the spleens of the 4T1.2 bearing mice were very large. The 4T1 cells produce copious quantities of G-CSF which would account for this. It is also known that MKs can be found in the spleen as well as in the bone. Therefore, we counted the numbers of MKs in the spleen (Figure 8).

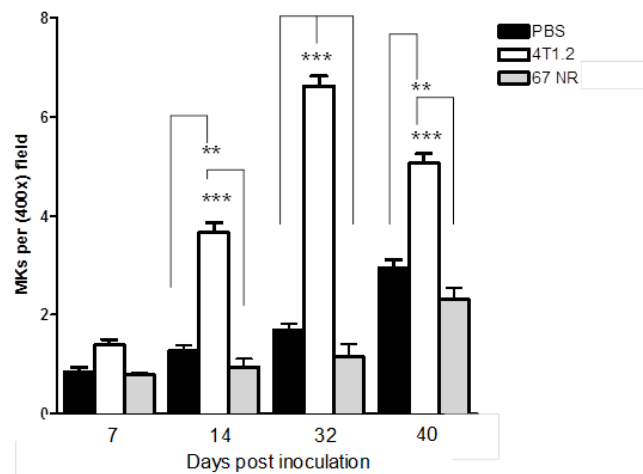


Figure 8. Spleens from mice inoculated with 4T1.2, 67NR or PBS as a control. Spleens from mice injected with 4T1.2 had significantly more MKs than the other groups. Spleens from 4-6 mice were harvested 7, 14, 32, or 40 days following mammary gland inoculation of 4T1.2, 67NR or PBS, fixed and prepared for IHC. MKs (vWF+) were counted in 30 fields per spleen at 400X field +/- S.E.M. ** $P < 0.01$, $P < 0.001$.

3. TPO and SDF-1

Sera from each group of mice, 4T1.2, 67NR and PBS injected, were tested for TPO and for SDF-1 using an ELISA (Figure 9). The detected values were within the normal ranges for mice. However, the mice injected with 67 NR showed increased levels of TPO compared the other groups at all times tested. At 40 days post inoculation, the 4T1.2 mice had significantly lower concentrations of TPO than the PBS or 67 NR groups. SDF-1, which is important for the movement of MKs to the blood vessels, showed reduced values in both groups of cancer treated mice later post inoculation.

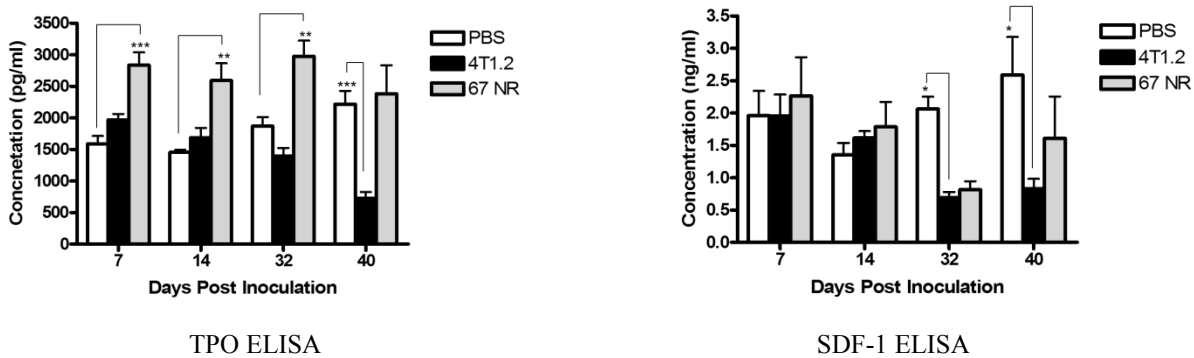


Figure 9. TPO and SDF-1 concentrations in the sera of Balb/c mice inoculated with 4T1.2, 67NR or with PBS. At various times following mammary gland inoculation of 4T1.2, 67NR or PBS, serum was taken and analyzed by ELISA for TPO or SDF-1. Shown are the means +/- SD for six mice per group. Statistically significant differences are indicated between groups. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The average reported range for SDF-1 levels in mice is 1.46-6.84ng/mL; the average values for TPO is in the range of 2972-6645pg/mL.

4. Blood cells

We counted the white blood cells of the mice at the time of sacrifice (Figure 10). The mice inoculated with 4T1.2 but not with 67 NR (the non-metastatic variant) or with PBS showed an almost 10 fold increase in white blood cells. This increase was due mainly to an increase in granulocytes.

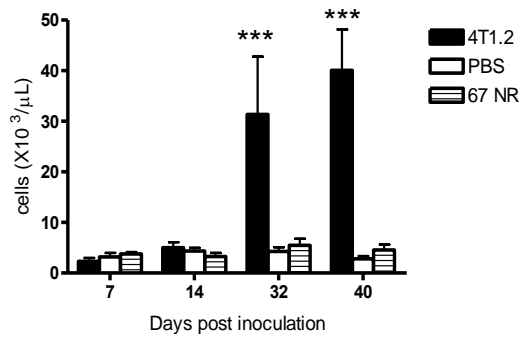


Figure 10. White Blood Cell counts from mice following inoculation of 4T1.2-luc cells, 67NR cells, or PBS into the mammary gland (MG). Blood was collected at the time of sacrifice as indicated. Shown are the means \pm SD for six mice per group. *** $P < 0.001$ 4T1.2 D 32 & 40 are significantly higher than PBS D 32 & 40.

Task 5. Metastasis in TPO knockout model.

We reasoned that if MKs were important for metastasis then mice with few MKs would be more resistant to metastasis. It was necessary to transfer the TPO^{-/-} genotype to the Balb/c background. This task is complete (see Task 1 above). We received two mating pairs of heterozygote, TPO^{+/-} on the Balb/c background from Jackson Laboratory early in April, 2013. We began breeding the mice to obtain the homozygotes and to inject them with 4T1.2 cells (Table 1).

Table 1: Summary of injection of TPO knockout mice with 4T1.2 cancer cells			
Dates inoculated with 4T1.2	-/-	+/-	+/+
12-2-13	2	2	2
1-27-14	2	3	0
3-13-14	2	2	0
3-31-14	2	0	0
5-6-14	2	4	0
Total inoculated	10	11	2

Heterozygous, TPO^{+/-}, were bred to obtain homozygous, TPO^{-/-} mice. Females were injected in the 4th mammary gland as they reached approximately 8-12 weeks of age. The dates and numbers of mice injected are given. For comparison, we also tested heterozygotes and wild type mice.

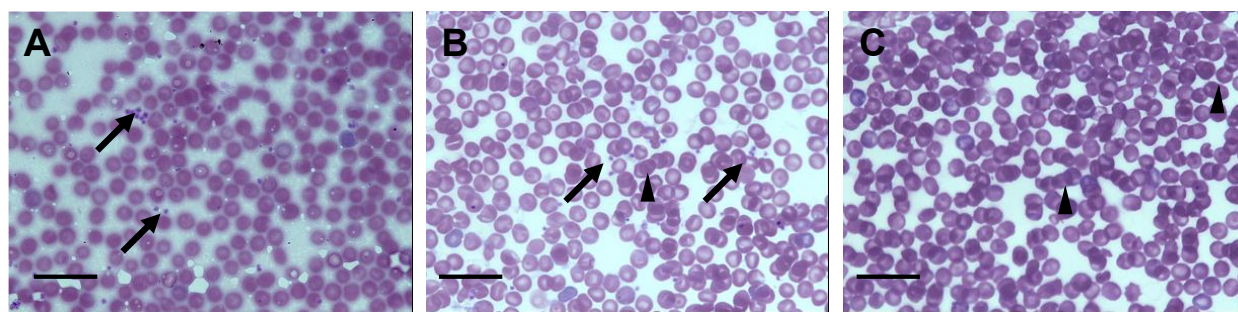


Figure 11. Images of blood smears from wild type (A), heterozygotes (B) and TPO^{-/-} mice. Arrows point to platelets. Arrowheads point to rouleaux. Scale bar indicates .

Blood smears were prepared from the three groups of mice (Figure 11). It was more difficult to find platelets in the heterozygous or knockout mice (arrows). When the blood was analyzed in the Hemavet instrument, the rbc mean volume was increased in the TPO^{-/-} mice. We have also measured platelet numbers using the Hemavet System (Figure 12). The platelet counts were not significantly different among groups. However, in the blood smears it was difficult to find platelets in samples from the TPO^{-/-} mice.

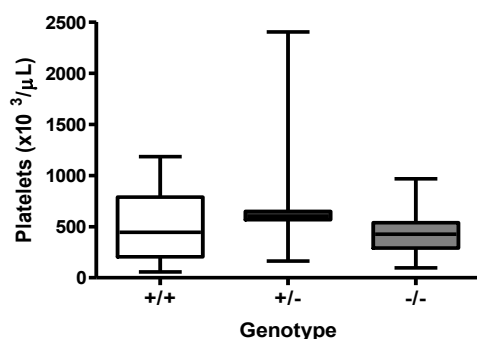


Figure 12. Platelet counts for wild type, heterozygous and TPO knockout mice. Blood samples were drawn from wild type, heterozygous and TPO knockout mice. Platelet counts were determined using the Hemavet 950FS. *n* = 26, 18 and 9 for +/+, +/- and -/-, respectively.

Due to the time constraints and the scarcity of the female knockout mice, we inoculated the mice as they became available (Table 1). PCR was used to determine whether the mice were KO, heterozygous or wild type. The TPO KO did not breed more slowly than the others nor did they have any apparent symptoms. TPO concentrations in the serum of the TPO^{-/-} were below the levels of detection (Table 2). Thus far, we have noted that the TPO^{-/-} mice develop metastasis sooner than the wild type or the heterozygous mice (Figure 13).

Table 2. Serum concentrations of TPO in wild type (+/+), heterozygous (+/-) and knockout (-/-) mice. Sera were analyzed by ELISA. Values are mean pg/ml +/- SEM. Calculated value below the limits of detection. N shown in ().

Wild type	(4)	573.7 +/- 54.3
Heterozygous	(12)	293.7 +/- 49.4
Knockout	(9)	31.2*

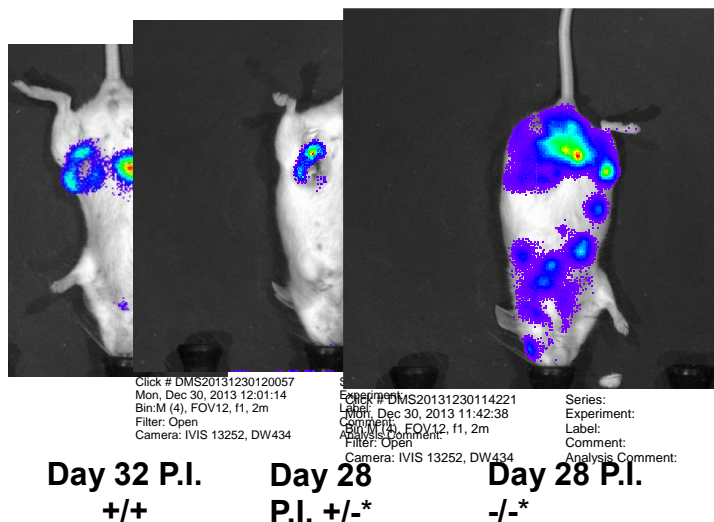


Figure 13. Comparison of metastases in TPO^{-/-}, heterozygous and wild type mice. Female mice, 13 weeks, were injected into the fourth mammary gland with 4T1.2 cells. The mice were imaged at the days shown post injection. The TPO^{-/-} was sacrificed on 28. The +/- was sacrificed on day 37, and the wild type on day 40.

We have fixed the femurs and spleens of all the mice tested. They will be sectioned and stained for MKs. Walter Jackson will be completing this work this summer as part of his Ph.D. Study.

Task 6. Alternative to Task 5. If for some reason it appears that we are unable to breed the congenic TPO^{-/-} mice, we will use the wild type Balb/c and treat them with an Anagrelide metabolite to reduce the numbers of megakaryocytes.

Given that the congenic TPO^{-/-} mice were bred, this alternative task was not necessary.

Supporting *in vitro* experiments. In order to more clearly define the interactions between MKs and the cells of the bone metastatic environment, several *in vitro* experiments were carried out. We hypothesized that cancer cells and/or osteoblasts would affect MK proliferation, differentiation or migration. These data were included in a MS thesis in Physiology which was successfully defended in July 2013 by Paige Chandler. A Ms has been prepared and will be submitted for publication after it is edited.

1. The effect of cancer cell condition medium on the proliferation, differentiation and migration of a megakaryocyte line (MEG-01).

In the first series of experiments, conditioned medium was prepared from MDA-MB-231 and metastatic suppressed variant, MDA-MB-231BRMS1 cells and added to MEG-01 cells to test the effect of the conditioned medium on the proliferation (MTT assay), differentiation (formation of multinucleated cells) and migration (transwell assay) of the MK line. Compared to normal growth medium, RPMI-1640 plus 10% serum, the conditioned media had little effect on the proliferation of the MEG-01 except at 72 hrs when proliferation was suppressed (Figure 14). In contrast, the number of multinucleated MEG-01s increased over the same cells maintained in standard growth medium and to the same level as MEG-01s exposed to the phorbol ester PMA at 10 ng/ml (Figure 15). Migration of MEG-01s towards the conditioned medium was not significantly increased (Figure 16).

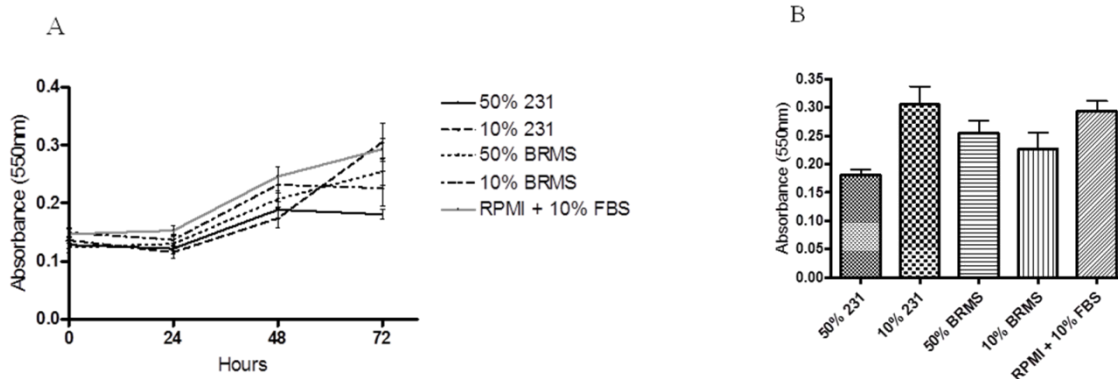


Figure14: Cancer cell conditioned medium decreased proliferation of Meg-01 cells in vitro. Conditioned medium was prepared from MDA-MB-231 (231) metastatic breast cancer cells and from the MDA-MB-231BRMS1 variant (BRMS). CM was added at 10 or 50% concentrations to Meg-01 cells at the time of plating (5,000/well, 96 well plates). Cultures were maintained at 37° C in 5% CO₂ in a humidified incubator. At times indicated, a culture plate was removed and the cell numbers determined by an MTT assay. **A.)** Cell proliferation over time. **B.)** Comparison of cell proliferation at 72 hrs. Results represent pooled data from 3 experiments. Treatments were done in quadruplicate (n = 12). Error bars represent mean ± SEM. Values were compared using a two-way ANOVA with Dunnett's test. * = $p < 0.05$, *** = $p < 0.001$ compared to RPMI-1640 + 10% FBS (control).

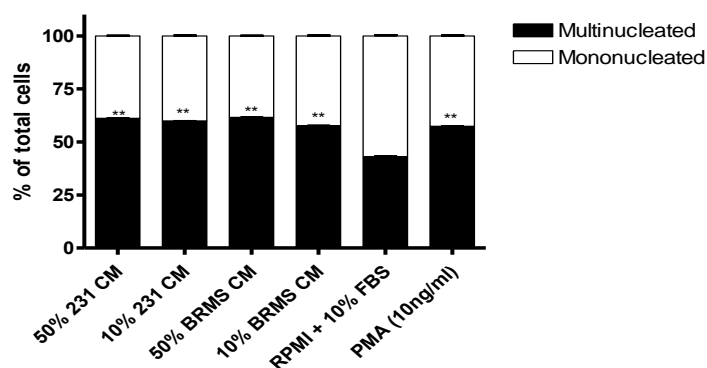


Figure 15: Cancer cell conditioned medium increased differentiation of Meg-01 cells in vitro. Conditioned medium (CM) was prepared from MDA-MB-231 metastatic breast cancer cells and from the MDA-MB-231BRMS1 variant. CM was added at 10 or 50%, as indicated, to Meg-01 cells at the time of plating (20,000/well, 24well plate). PMA (10 ng/ml) was added as a positive control. RPMI-1640 complete medium was used as a negative control. Cultures were maintained at 37° in 5% CO₂ in a humidified incubator. After 3 days, attached cells and cells cytocentrifuged from the culture medium were fixed and stained with Giemsa. A cell was considered differentiated if it contained more than one nucleus. Twenty fields each (about 100-150 cells) for cells from the supernatant and attached cells were counted at 40X for each treatment. Results represent pooled data from 3 experiments. Each treatment was done in

triplicate ($n = 9$). Error bars represent mean \pm SEM. Values were compared using a one-way ANOVA with Dunnett's test. ** = $p < 0.01$, compared to the negative control.

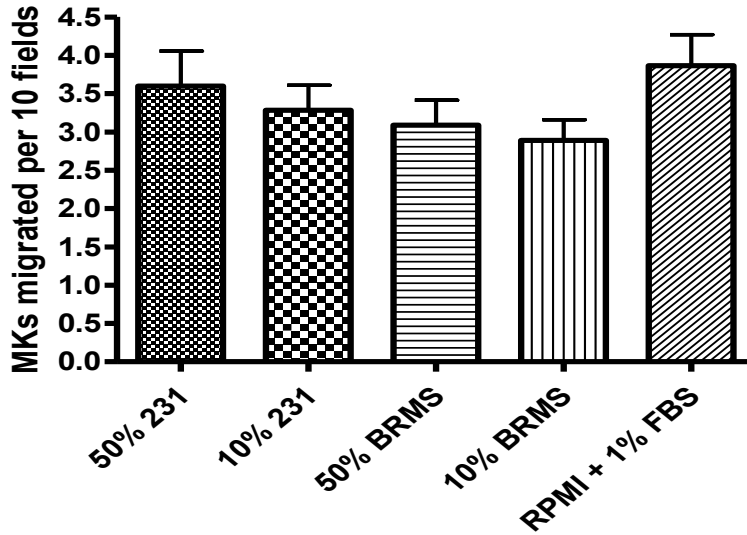


Figure 16. Cancer cell conditioned medium had no effect on the migration of MEG-01 cells. Conditioned medium (CM) was prepared from MDA-MB-231 metastatic breast cancer cells and from the MDA-MB-231BRMS1 variant. CM from either cell line was added to the bottom chamber of a transwell plate at 10 or 50%. Meg-01 cells were then plated (100,000 cells/well) in the top of the 24 well transwell plate. Cultures were maintained at 37° C in 5% CO₂ in a humidified incubator. Following an 8 hr incubation, the membranes were collected, washed, fixed, stained with Giemsa, and mounted on microscope slides. Cells on the underside of the membrane were counted in 10 random fields at 40X magnification. Results represent pooled data from 3 experiments. Each experiment generated triplicate samples ($n = 9$) except in one case, $n = 8$. Error bars represent mean \pm SEM. Values were compared using a one-way ANOVA with Dunnett's test.

2. The effect of conditioned medium from osteoblasts/cancer cells on the proliferation, differentiation and migration of MEG-01 cells.

Megakaryopoiesis is believed to be controlled by the endosteal osteoblastic niche. Cancer cells cause osteoblasts to produce inflammatory cytokines. Therefore, we proposed that the combination of cancer cells and osteoblasts could also affect MEG-01s. We treated human primary osteoblasts, NHost (Lonza), with 50% conditioned medium from MDA-MB-231 or MDA-MB-231BRMS1 for 24 hours. This “double” conditioned medium was collected and added to MEG-01. Their growth in the various conditioned media was compared to that in RPMI-1640 with 10% FBS (Figure 17). The medium from NHost cells alone increased proliferation almost two-fold over the standard growth medium. The double conditioned medium also increased growth to the same extent.

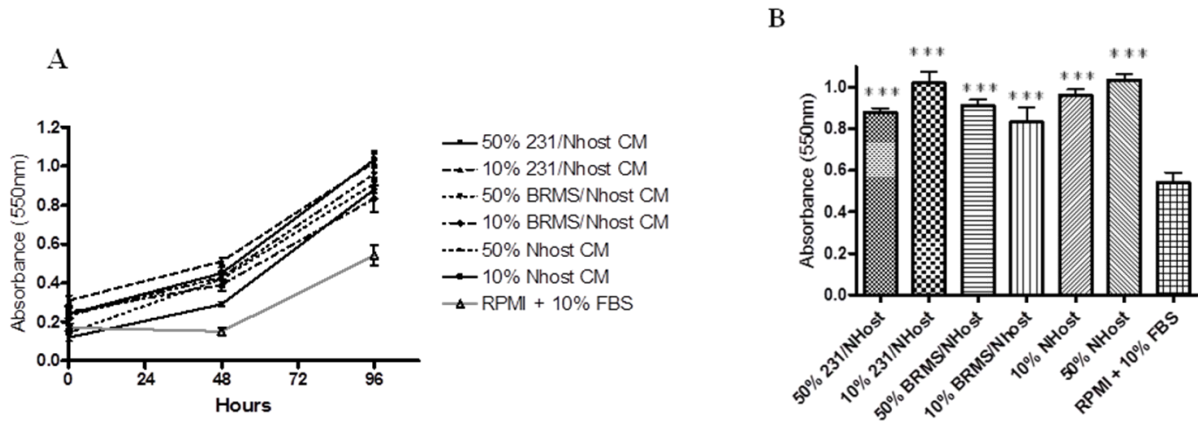


Figure 17. Osteoblast and cancer cell conditioned medium increased proliferation of Meg-01 cells in vitro. Conditioned medium was prepared from MDA-MB-231 cancer cells and from the MDA-MB-231BRMS1 variant. Cancer cell CM was then added at 50% to mature NHost cells. After 24 hrs. this double CM was collected and added to Meg-01 cells at 10 or 50% as indicated. Meg-01 cells were plated (5,000/well, 96 well plate). Cultures were maintained at 37° C in 5% CO₂ in a humidified incubator. At times indicated, a culture plate was removed and the cell numbers determined by the MTT assay. **A.)** Cell number over time. **B.)** Comparison of cell proliferation at 144 hrs. Results represent pooled data from 3 experiments. Treatments were done in quadruplicate (n = 12). Error bars represent mean \pm SEM. Values were compared using a two-way ANOVA with Dunnett's test. *** = $p < 0.001$ compared to RPMI-1640 + 10% FBS, the negative control.

The double conditioned media had little effect on migration (Figure 18). The 50% conditioned medium from the MDA-MB-231BRMS, NHOST combination statistically increased MEG-01 migration above that of growth medium. However, the number of migrated cells was very small in all cases.

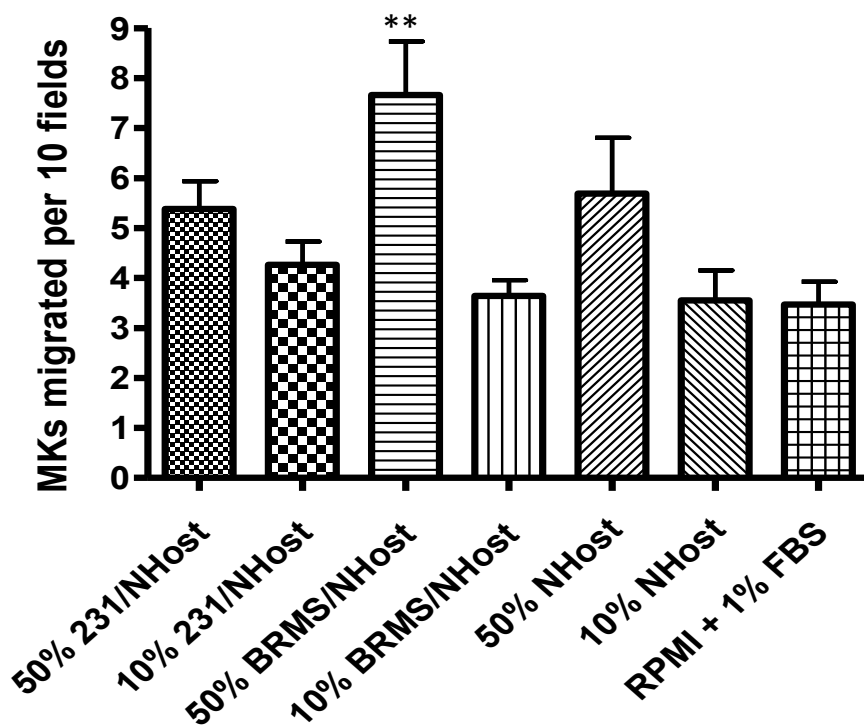


Figure 18. Osteoblast/cancer cell conditioned medium had little effect of migration of Meg-01 cells in vitro. Double Conditioned medium prepared by treating NHost cells with CM from MDA-MB-231 or from MDA-MB-231BRMS1 was added to the bottom chamber of a transwell plate at 10 or 50%. Meg-01 cells were then plated (100,000 cells/well) in the top of the 24 well transwell plate. Cultures were maintained at 37° C in 5% CO₂ in a humidified incubator. Following an 8 hr incubation, the membranes were collected, washed, fixed, stained with Giemsa, and mounted on microscope slides. Cells on the underside of the membrane were counted in 10 random fields at 40X magnification. Results represent pooled data from 3 experiments. Each experiment generated triplicate samples (n= 9) except in one case, n=8. Error bars represent mean \pm SEM. Values were compared using a one-way ANOVA with Dunnett's test. **= $p < 0.01$. compared to RPMI-1640 + 10% FBS, the negative control.

Thus it appeared that the conditioned medium from the combination of cancer cells and osteoblasts affected MEG-01 proliferation, and differentiation.

3. The effect of conditioned medium from MEG-01 cells on MDA-MB-231 or on MDA-MB-231BRMS1 cancer cells.

We also tested whether conditioned media prepared from MEG-01 affected the proliferation or migration of MDA-MB-231 or MB-231BRMS1. MEG-01 were either differentiated in the presence of PMA or left undifferentiated. The conditioned medium from the megakaryocyte line had very little effect on cancer cell proliferation compared to their growth in DMEM, 5% FBS, except that 10% conditioned medium from undifferentiated MEG-01 caused a small but significant decrease (Figure 19). There was no effect on migration of MDA-MB-231 cells (Figure 20).

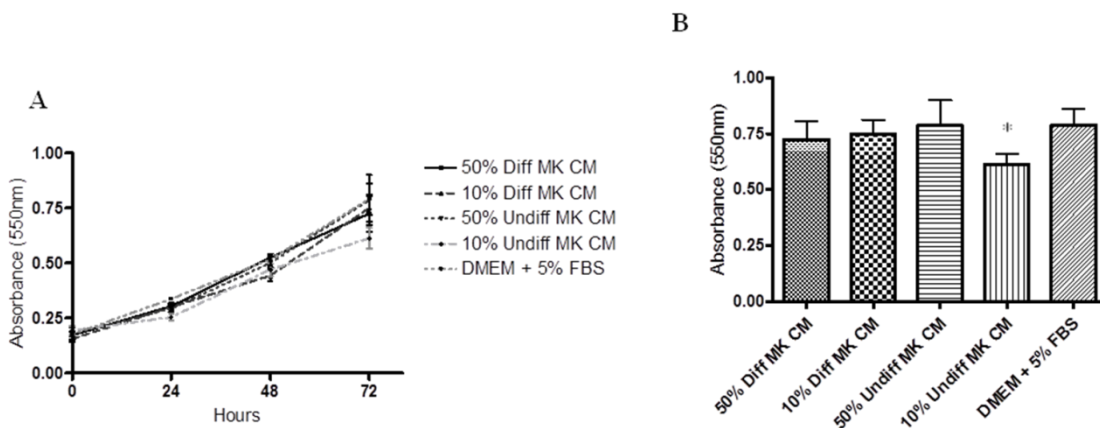


Figure 19. MEG-01 CM had no effect on proliferation of MDA-MB-231 cells in vitro. Conditioned medium was prepared from differentiated (treated with 10ng/ml PMA) or undifferentiated MEG-01 cells. CM, 10 or 50%, or normal growth medium (control) was added to MDA-MB-231 cells plated (5,000/well, 96 well plate). Cultures were maintained at 37° C in 5% CO₂ in a humidified incubator for 72 hrs. At times indicated on the x axis (every 24 hrs.), a plate was removed from the incubator and the cell numbers determined by the MTT assay. A.)

Cell (MDA-MB-231) number indicated by absorbance, over time. **B.)** Cell number at 72 hrs. Results represent pooled data from 3 experiments. Treatments were done in triplicate ($n = 12$). Error bars represent mean \pm SEM. Values were compared using a two-way ANOVA with Dunnett's test. * = $p < 0.05$ compared to DMEM + 5% FBS, the negative control.

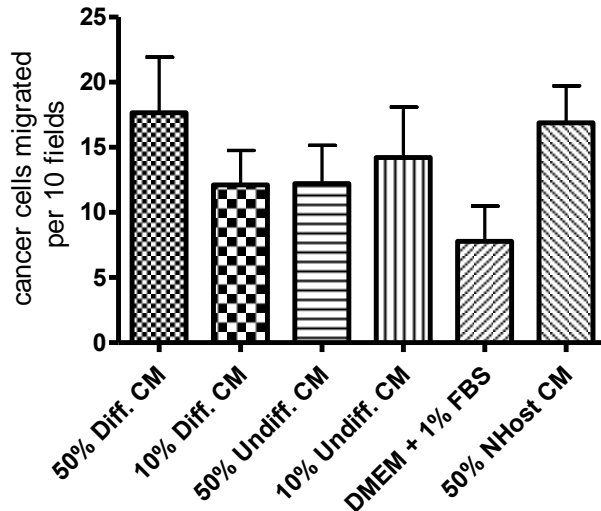


Figure 20. MEG-01 conditioned medium had no effect on MDA-MB-231 cancer cell migration. Conditioned medium prepared from differentiated (treated with 10ng/ml PMA) or undifferentiated MEG-01 cells was added to the bottom chamber of a transwell plate at 10 or 50%. MDA-MB-231 cells were then plated (5,000/well, 96 well plate) in the top of the 24 transwell plate. 50% NHost CM was used as a positive control. Cultures were maintained at 37°C in 5% CO₂ in a humidified incubator. Following an 8 hr incubation, the membranes were collected, washed, fixed, stained with Giemsa, and mounted on microscope slides. Cells on the underside of the membrane were counted in 10 random fields at 40X magnification. Results represent pooled data from 3 experiments. Treatments were done in triplicate ($n = 9$). Error bars represent mean \pm SEM. Values were compared using a one-way ANOVA with Dunnett's test.

Similarly, conditioned medium from MEG-01 did not affect the proliferation of MDA-MB-231BRMS1 (Figure 20) or their migration (Figure 21).

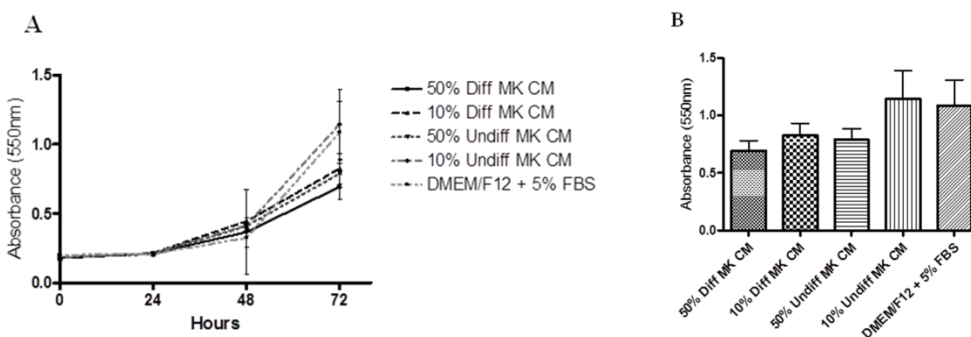


Figure 21. MEG-01 CM had no effect on proliferation of MDA-MB-231BRMS1 cells in vitro. Conditioned medium was prepared from differentiated (treated with 10ng/ml PMA) or undifferentiated MEG-01 cells. Meg-01 cells were then plated (5,000/well, 96 well plate). Cultures were maintained at 37° C in 5% CO₂ in a humidified incubator. At times indicated, a culture plate was removed and the cell numbers determined by the MTT assay, also described in the methods section. **A.)** Cell (MDA-MB-231BRMS1) number over time. **B.)** Cell proliferation at 72 hours. Results represent pooled data from 3 experiments. Treatments were done in quadruplicate (n = 12). Error bars represent mean ± SEM. Values were compared using a two-way ANOVA with Dunnett's test.

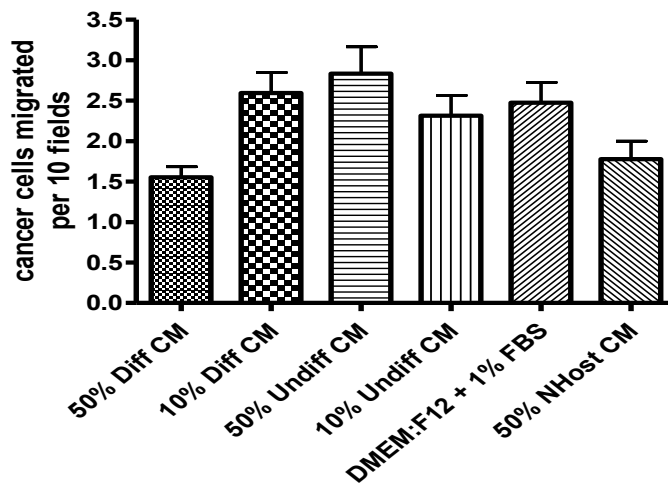


Figure 22. MEG-01 conditioned medium had no effect on MDA-MB-231BRMS1 cancer cell migration. Conditioned medium prepared from differentiated (treated with 10ng/ml PMA) or undifferentiated MEG-01 cells was added to the bottom chamber of a transwell plate at 10 or 50%. MDA-MB-231BRMS1 cells were then plated (5,000/well, 96 well plate) in the top of the 24 transwell plate. 50% NHost CM + 10% FBS was used as a positive control. Cultures were maintained at 37° C in 5% CO₂ in a humidified incubator. Following an 8 hr incubation, the membranes were collected, washed, fixed, stained with Giemsa, and mounted on microscope slides. Cells on the underside of the membrane were counted in 10 random fields at 40X magnification. Results represent pooled data from 2 experiments. Treatments were done in triplicate (n = 6) except for the 50% Nhost treatment (positive control) which had an n = 3. Error bars represent mean ± SEM. Values were compared using a two-way ANOVA with Dunnett's test.

Cancer cells were examined for any changes in morphology brought about by exposure to conditioned media from MEG-01. None were seen (data not shown).

Task 7. Data analysis. Manuscript preparation .

Two graduate students carried out this work as part of their theses. A Ms thesis in Physiology was completed and submitted by Paige Chandler in 2013. She has prepared a manuscript that will be submitted for publication after editing. A PhD thesis is being prepared by Walter Jackson

III. His thesis defense is anticipated in the fall of 2014. He will prepare a manuscript in parallel with his thesis.

KEY RESEARCH ACCOMPLISHMENTS

- Successful re-derivatization of TPO^{-/-} mice and transfer to a BALB/c background. This work is complete. Breeding pairs were received in April of 2013 and were used to produce heterozygotes, TPO^{-/+} and homozygotes, TPO^{-/-}.
- Completion of animal inoculations intracardiac and into mammary gland; images taken; all femurs have been sectioned, stained for von Willebrand's and the megakaryocytes counted.
- Distinguished between a model in which the cancer cells send a systemic signal to increase megakaryopoiesis and a model in which cancer cells already in the bone cause an increase in megakaryocytes.
- Successfully assayed for SDF-1 and thrombopoietin in the serum of mice inoculated with cancer cells.
- Completed the syngeneic experiments with 4T1.2 cells and bone metastases.

REPORTABLE OUTCOMES

Manuscripts

No completed manuscripts at this time. Manuscript in preparation.

Thesis

Chandler, Paige N. 2013. In Vitro Interactions Among Breast Cancer Cells, Osteoblasts and Megakaryocytes. A MS thesis in Physiology. The Pennsylvania State University.

Book chapter

Jackson, W. A., V. Krishnan, and A. M. Mastro. 2012. The Breast Cancer Metastasis in Bone Conundrum in Metastatic Cancer: Integrated Organ System and Biological Approach. Ed. R. Jandial and K. Hunter. Landes Bioscience Madame Curie Data Base.

Abstracts

Mastro, A. M., D. M. Sosnoski and W. A. Jackson (2011). The Role of Megakaryocytes in Breast Cancer Metastasis to Bone. ERA of Hope Meeting #2603, Orlando, Florida, August 2-5.
Walter Jackson III (2012). The Role of Megakaryocytes in Breast Cancer Metastasis to Bone. Penn State University Graduate Student Exhibition.

Walter Jackson III (2013) The Role of Megakaryocytes in Breast Cancer Metastasis to Bone. The Annual Meeting of the American Association for Cancer Research, April 6-10, 2013, Washington, D.C.

Paige Chandler, Walter Jackson, Donna Sosnoski, Andrea M Mastro. 2014. In Vitro Interactions Among Breast Cancer Cells, Osteoblasts and Megakaryocytes. The Graduate Exhibition, Penn State University, April 6.

Paige Chandler, Walter Jackson, Donna Sosnoski, Andrea M Mastro. 2013. In Vitro Interactions Among Breast Cancer Cells, Osteoblasts and Megakaryocytes. Sensing and Signaling Across the Mucosa: From Homeostatis to Pathogenesis. The Summer Symposium. Penn State University, June 6.

Training

Several undergraduate students have worked on aspects of this project. A rotating graduate student also was involved.

Maya Evanitsky, a major in Forensic Sciences, Chemistry option, was involved with staining and counting megakaryocytes.

Nick Kendersky was a Penn State Biochemistry and Molecular Major. He learned all of the histological protocols and helped with fixation, sectioning and IHC. He is now working at the Oregon Health Sciences University.

William Turbitt, a Physiology Graduate student, who rotated in the laboratory, sectioned and stained spleens. He generated the data regarding megakaryocytes in the spleen. He is now a graduate student in the Nutrition Program at Penn State.

Shelby Foster, a sophomore major in Biochemistry and Molecular Biology is a WISER student in the laboratory. She spent several weeks working with Paige Chandler on the *in vitro* studies.

Paige Chandler, who is completing her second year as a Physiology graduate student, has been working on the *in vitro* aspect of this project. She has begun to explore the factors secreted by megakaryocytes, osteoblasts and cancer cells in culture and how they may affect one of the other cell types. She received a Sigma Xi research grant (\$1000) to help support her *in vitro* studies. She successfully defended her MS thesis in Physiology and passed her candidacy exam. She will stay as a PhD student in the Physiology Program

Walter Jackson III has carried out the bulk of the work on this project. Walter joined the laboratory as a Ph.D. student in 2010. He has an MS from Alcorn University through the Bridges program with Penn State. Walter inoculated athymic mice in the heart and mammary gland. He carried out IVIS imaging, sacrificed mice and harvested femurs. He has fixed and processed the bones and prepared the sections. He has learned how to carry out the immunohistochemistry. He also is breeding the TPO^{-/-} mice. He presented his data at the 2013 national AACR meeting. He also received the 2012-2013 Robert T. Simpson Innovation Award in the Biochemistry and Molecular Biology Department at Penn State. He will include the data in his thesis.

In the summer of 2013, Ashaki Nehisi, worked on the project as a Summer Research Opportunity Student. She was a junior from the University of Baltimore. She helped optimize cytokeratin staining of bone sections.

CONCLUSIONS

We had noted an increase in MKs in the bones of mice with metastatic breast cancer in a xenograft model. We hypothesized that either the cancer cells sent a signal to the bone with the resulting increase in MKs or that MKs increased because of factors from cancer cells or induced by cancer cells in the bone. Using a xenograft model we found that the increase in MKs only occurred with a metastatic model. It did not occur when the cancer stayed localized to the breast. In the metastatic model, the increase in MKs was seen late in the metastatic process suggesting that the MKs increased under the influence of the cancer cells and not vice versa.

Thromboembolism is one of the most common causes of death in cancer patients [4]. Indeed increased platelets are a poor prognostic factor for breast cancer metastasis [5]. Consistent with this information is the observation that we made in a mouse model, that MKs were increased in the marrow of metastasis bearing mice [1]. We speculated that MKs either result from bone metastasis or play a role in allowing tumor colonization of bone. We designed animal experiments to test between these two possibilities. Task 2 and 3 are complete. Breeding of the TPO^{-/-} mice is on track and we have tested the TPO^{-/-} for metastasis. Our data suggest that the TPO^{-/-} mice are more susceptible to metastasis than the wild type mice.

REFERENCES

1. Bussard KM, Venzon DJ, Mastro AM (2010) Osteoblasts are a major source of inflammatory cytokines in the tumor microenvironment of bone metastatic breast cancer. *J Cell Biochem* 111: 1138-1148. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=20683902
2. Paapadantonakis N, Ravid K (2009) Development of Megakaryocytes. In: Wicrema A, Kee B, editors. *Molecular Basis of Hematopoiesis*: Springer Science+Business Media. pp. 95-126.
3. Zhang XH, Wang Q, Gerald W, Hudis CA, Norton L, et al. (2009) Latent bone metastasis in breast cancer tied to Src-dependent survival signals. *Cancer Cell* 16: 67-78. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19573813
4. Trikha M, Nakada MT (2002) Platelets and cancer: implications for antiangiogenic therapy. *Semin Thromb Hemost* 28: 39-44. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11885024
5. Taucher S, Salat A, Gnant M, Kwasny W, Mlineritsch B, et al. (2003) Impact of pretreatment thrombocytosis on survival in primary breast cancer. *Thromb Haemost* 89: 1098-1106. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12783124